Exhibit F

RAPID COMMUNICATION

The Gene for the Human Erythropoietin Receptor: Analysis of the Coding Sequence and Assignment to Chromosome 19p

By John C. Winkelmann, Laura A. Penny, Larry L. Deaven, Bernard G. Forget, and Robert B. Jenkins

The full-length coding sequence of the human erythropoietin receptor has been assembled from cDNA and genomic DNA. The derived 508 amino acid sequence is 82% identical to the murine erythropoietin (Epo) receptor with one single residue gap in alignment. There is no major structural difference between the human and murine receptor molecules. Nucleotide sequence homology is, as expected, very high within the coding domain. Unexpectedly, there are two distinct, short stretches of 3′ untranslated sequence homology between human and murine cDNAs. The functional significance of this sequence conservation is unknown. The human Epo receptor gene is localized to human chromosome 19p by in situ hybridization. This chromosome assignment is confirmed by hybridization to a panel of sorted human chromosomes.

ERYTHROPOIETIN (Epo) is a glycoprotein hormone that regulates erythropoiesis.[1,2] Its synthesis by the kidney is stimulated by decreased oxygen delivery. Epo exerts its effect on early erythroid progenitors, resulting in cell proliferation and erythroid differentiation. The molecular mechanism of Epo action is largely unknown. In recent years it has become apparent that, like other glycoprotein hormones, the biologic effects of Epo are initiated by its association with a specific cell-surface receptor.[3]

The recent expression cloning of the murine Epo receptor cDNA has made direct examination of the Epo receptor molecule possible.[4] The reported cDNA clone encodes a 55-Kd peptide with a single putative transmembrane domain approximately in the middle of the molecule. The Epo receptor belongs to a previously unrecognized family of growth factor receptors; it is structurally related to the interleukin-2 (IL-2) receptor β subunit[5-7] and the receptors for IL-3,[8] IL-4,[9,10] IL-6,[6] and granulocyte-macrophage colony-stimulating factor (GM-CSF).[11] Many important questions remain regarding subunit composition, hormone binding characteristics, and signal transduction properties of the Epo receptor.

The human Epo receptor is of interest to investigators studying erythropoiesis in humans and the biology of clinical disorders such as pure red blood cell (RBC) aplasia and polycythemia vera. To determine the structure of the human receptor, we have isolated and characterized a cDNA clone encoding 75% of the human fetal liver Epo receptor using synthetic oligonucleotides derived from the reported murine Epo receptor. Using the nucleotide sequence of this cDNA and exon sequence derived from 5′ genomic DNA, we have derived the primary structure of the human Epo receptor. Human chromosome assignment and sublocalization have been performed.

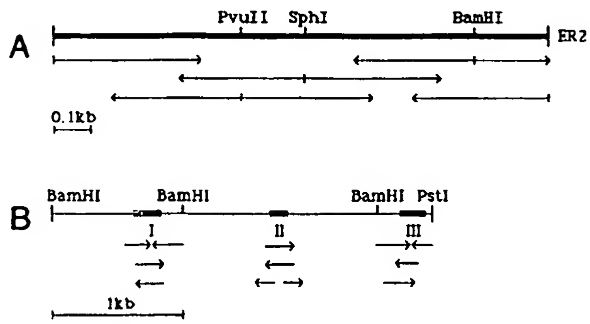

Fig 1. Molecular clones containing the human Epo receptor coding sequence. The relative scale, in kilobases (kb), is shown at the bottom of each. (A) The sequencing strategy used to analyze cDNA clone ER2 is depicted. Map sites of restriction enzymes used to make sequencing templates are shown. Arrows designate individual sequencing reactions. (B) The sequencing strategy used to analyze the 5′ exons of the chromosomal gene is shown. The positions of exons I through III (boxes) are labeled. The 5′ extent of exon I is not known. The known 5′ untranslated region is indicated by an open box. Arrows show individual sequencing reactions.

MATERIALS AND METHODS

Molecular cloning. Two synthetic 36-base oligonucleotides, based on the 3′ coding sequence of the reported murine Epo receptor, were used as hybridization probes. These oligonucleotides (A: 5′-GATGGCCCCTACTCCCACCCCTATGAGAACAGCCTT-3′, a sense probe; and B: 5′-CACATAGCCGGGATGCAGAGGCTCT-GAGTCTGGGAC-3′, an antisense probe) were labeled by reacting 1 μg of DNA with α-^{32}P-deoxycytidine triphosphate (Amersham, Arlington Heights, IL) in the presence of terminal deoxynucleotidyl transferase (Pharmacia, Piscataway, NJ). Oligonucleotide A was hybridized to one million bacteriophage plaques plated from a human fetal liver cDNA library[12] constructed in λgt11 and transfered to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Hybidization conditions wre 70 mmol/L Tris pH 7.4, 6X SSC (1X SSC; 0.15 mol/L NaCl, 0.015 mol/L Na citrate), 5X Denhardts (1X Denhardts: 0.02 gram% each of Ficoll, polyvinylpyrrolidone, and fraction V bovine serum albumin), 100 μg/mL denatured

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1     TGGGCTCCCC GTGGCGGGGG CTGTATCATG GACCACCTCG GGGCGTCCCT
51    CTGGCCCCAG GTCGGCTCCC TTTGTCTCCT GCTCGCTGGG GCCGCCTGGG

101   CGCCCCCGCC TAACCTCCCG GACCCCAAGT TCGAGAGCAA AGCGGCCTTG
151   CTGGCGGCCC GGGGGCCCGA AGAGCTTCTG TGCTTCACCG AGCGGTTGGA
201   GGACTTGGTG TGTTTCTGGG AGGAAGCGGC GAGCGCTGGG GTGGGCCCGG

251   GCAACTACAG CTTCTCCTAC CAGCTCGAGG ATGAGCCATG GAAGCTGTGT
301   CGCCTGCACC AGGCTCCCAC GGCTCGTGGT CGGGTGCGCT TCTGGTGTTC
351   GCTGCCTACA GCCGACACGT CGAGCTTCGT GCCCCTAGAG TTGCGCGTCA
401   CAGCAGCCTC CGGCGCTCCG CGATATCACC GTGTCATCCA CATCAATGAA

451   GTAGTGCTCC TAGACGCCCC CGTGGGGCTG GTGGCGCGGT TGGCTGACGA
501   GAGCGGCCAC GTAGTGTTGC GCTGGCTCCC GCCGCCTGAG ACACCCATGA
551   CGTCTCACAT CCGCTACGAG GTGGACGTCT CGGCCGGCAA CCGGCCAGGG
601   AGCGTACAGA GGGTGGAGAT CCTGGAGGGC CGCACCGAGT GTGTGCTGAG
651   CAACCTGCGG GGCCGGACGC GCTACACCTT CGCCGTCCGC GCGCGTATGG
701   CTGAGCCGAG CTTCGGCGGC TTCTGGAGCG CCTGGTCGGA GCCTGTGTCG
751   CTGCTGGAGC CTAGCGACCT GGACCCCCTC ATCCTGACGC TCTCCCTCAT
801   CCTCGTGGTC ATCCTGGTGC TGCTGACCGT GCTCGCGCTG CTCTCCCACC
851   GCCGGGCTCT GAAGCAGAAG ATCTGGCCTG GCATCCCGAG CCCAGAGAGC
901   GAGTTTGAAG GCCTCTTCAC CACCCACAAG GGTAACTTCC AGCTGTGGCT
951   GTACCAGAAT GATGGCTGCC TGTGGTGGAG CCCCTGCACC CCCTTCACGG
1001  AGGACCCACC TGCTTCCCTG GAAGTCCTCT CAGAGCGCTG CTGGGGGACG
1051  ATGCAGGCAG TGGAGCCGGG GACAGATGAT GAGGGCCCCC TGCTGGAGCC
1101  AGTGGGCAGT GAGCATGCCC AGGATACCTA TCTGGTGCTG GACAAATGGT
1151  TGCTGCCCCG GAACCCGCCC AGTGAGGACC TCCCAGGGCC TGGTGGCAGT
1201  GTGGACATAG TGGCCATGGA TGAAGGCTCA GAAGCATCCT CCTGCTCATC
1251  TGCTTTGGCC TCGAAGCCCA GCCCAGAGGG AGCCTCTGCT GCCAGCTTTG
1301  AGTACACTAT CCTGGACCCC AGCTCCCAGC TCTTGCGTCC ATGGACACTG
1351  TGCCCTGAGC TGCCCCCTAC CCCACCCCAC CTAAAGTACC TGTACCTTGT
1401  GGTATCTGAC TCTGGCATCT CAACTGACTA CAGCTCAGGG GACTCCCAGG
1451  GAGCCCAAGG GGGCTTATCC GATGGGCCCT ACTCCAACCC TTATGAGAAC
1501  AGCCTTATCC CAGCCGCTGA GCCTCTGCCC CCCAGCTATG TGGCTTGCTC
1551  TTAGGACACC AGGCTGCAGA TGATCAGGGA TCCAATATGA CTCAGAGAAC
1601  CAGTGCAGAC TCAAGACTTA TGGAACAGGG ATGGCGAGGC CTCTCTCAGG
1651  AGCAGGGGCA TTGCTGATTT TGTCTGCCCA ATCCATCCTG CTCAGGAAAC
1701  CACAACCTTG CAGTATTTTT AAATATGTAT AGTTTTTTTT TGTATCTATA
1751  TATATATATA CACATAAAAA AAAAAAAAA
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Fig 2. Assembled nucleotide coding sequence of the human Epo receptor. The coding sequence begins with ATG (underlined) at nucleotide 28. The position of each intron separating the 5′ exons is shown by an arrowhead. The termination codon (TAG) is underlined. The 22 nucleotides of the 3′ end of exon III are identical to the 3′ end of ER2.

herring sperm DNA, 100 μg/mL polydeoxycytidine, 0.01 mol/L sodium pyrophosphate, and 0.1% sodium dodecyl sulfate (SDS) at 50°C. Washing was performed to a maximum stringency of 6X SSC, 0.1% SDS, and 50°C. Four bacteriophage clones were positive with oligonucleotide A. These were counterscreened with oligonucleotide B. Each was weakly positive. The most intensely hybridizing clone (ER2) was selected for further analysis. The other three subsequently were shown to be false positives.

Genomic clones were isolated by plaque hybridization[13] from a bacteriophage λ Charon 4A library of total human DNA.[14] Exons were identified by hybridization to human cDNA, murine cDNA, and a 5′ synthetic oligonucleotide derived from the reported murine sequence (5′-ATGGACAAACTCAGGGTGCCCCTCTGGCCT-3′).[4] The murine cDNA was isolated by hybridization of oligonucleotides A and B (see above) to a cDNA library constructed in λgtll with RNA from a mouse erythroleukemia cell line.

Nucleotide sequence analysis. The ER2 cDNA and genomic DNA were subcloned into the plasmid pGEM7Z (Promega Biotec, Madison, WI) for restriction enzyme mapping and nucleotide sequence determination. The subcloning strategy used for sequence analysis is shown in Fig 1. The dideoxynucleotide method of Sanger et al[15] was used. Primers homologous to the SP6 and T7 bacteriophage promoters that flank the pGEM7Z polylinker were annealed to double-stranded plasmid templates. Sequenase (United States Biochemical, Cleveland, OH) was used for all sequencing reactions.[16] The labeling isotope was α-[35] S-thio-deoxytadenosine triphosphate. Deoxyinosine triphosphate was substituted for deoxyguanosine triphosphate in sequencing reactions to resolve sequence ambiguities.

Computer analysis of nucleotide sequence data was performed on microcomputer using The DNA Inspector IIe (TEXTCO, West Lebanon, NH) programs. Nucleotide and amino acid databank searches and alignments were accomplished on minicomputer using the Intelligenetics programs and GenBank database.

In situ hybridization to human metaphase chromosomes. The in situ hybridization method of Harper and Saunders,[17] as modified by Donlan,[18] was used. Fluorescent R-bands,[19] as well as routine Wright staining, were used to identify chromosomes.

Hybridization to sorted human chromosomes. Human metaphase chromosomes were isolated by flow-sorting.[20] They were spotted onto nitrocellulose discs, denatured, hybridized to ^{32}P-labeled ER2 cDNA as described,[21,22] and autoradiographed. The position of each sorted chromosome was marked on the filter discs. The authenticity of hybridization signals was established by superimposing the autoradiograph on the marked filters.

RESULTS

ER2 is a 1,347 base pair cDNA clone from human fetal liver. Its identification as an authentic Epo receptor clone

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HUM MDHLGASLWP QVGSLCLLLA GAAWAPPPNL PDPKFESKAA LLAARGPEEL LCFTERLEDL VCFWEEAASA
    || |   |||  || |||||| |||||| | | |||||||||| ||| || ||| |||| ||||| |||||||||
MUR MDKLRVPLWP RVGPLCLLLA GAAWAPSPSL PDPKFESKAA LLASRGSEEL LCFTQRLEDL VCFWEEAASS

71  GVGPGNYSFS YQLEDEPWKL CRLHQAPTAR GRVRFWCSLP TADTSSFVPL ELRVTAASGA PRYHRVIHIN
    |    ||||| |||| |  |  | |||||| | | |||||||| |||||||||| || || |||  ||||| ||||
    GMDF-NYSFS YQLEGESRKS CSLHQAPTVR GSVRFWCSLP TADTSSFVPL ELQVTEASGS PRYHRIIHIN

141 EVVLLDAPVG LVARLADESG HVVLRWLPPP ETPMTSHIRY EVDVSAGNRP GSVQRVEILE GRTECVLSNL
    |||||||| | | || | |   ||||||||||   ||| |||| |||||||||  |  |||| || ||||||||||
    EVVLLDAPAG LLARRAEEGS HVVLRWLPPP GAPMTTHIRY EVDVSAGNRA GGTQRVEVLE GRTECVLSNL

211 RGRTRYTFAV RARMAEPSFG GFWSAWSEPV SLLEPSDLDP LILTLSLILV VILVLLTVLA LLSHRRALKQ
    || ||||||| |||||||||  |||||||||  |||  ||||| ||||||||||  |  |||||| |||||| | |
    RGGTRYTFAV RARMAEPSFS GFWSAWSEPA SLLTASDLDP LILTLSLILV LISLLLTVLA LLSHRRTLQQ

281 KIWPGIPSPE SEFEGLFTTH KGNFQLWLYQ NDGCLWWSPC TPFTEDPPAS LEVLSERCWG TMQAVEPGTD
    |||||||||| |||||||||| |||||||| |  ||||||||    | |||||  ||||||  |     ||  || |
    KIWPGIPSPE SEFEGLFTTH KGNFQLWLLQ RDGCLWWSPG SSFPEDPPAH LEVLSEPRWA VTQAGDPGAD

351 DEGPLLEPVG SEHAQDTYLV LDKWLLPRNP PSEDLPGPGG SVDIVAMDEG SEASSCSSAL ASKPSPEGAS
    |||||||||| |||||||||| |||||||| |  || | |||| ||| | |||  || ||| | | |||| ||| |
    DEGPLLEPVG SEHAQDTYLV LDKWLLPRTP CSENLSGPGG SVDPVTMDEA SETSSCPSDL ASKPRPEGTS

421 AASFEYTILD PSSQLLRPWT LCPELPPTPP HLKYLYLVVS DSGISTDYSS GDSQGAQGGL SDGPYSNPYE
      |||||||| |||||| |   | |||||||| |||||||||| |||||||||| | |||  |   |||||| |||
    PSSFEYTILD PSSQLLCPRA LPPELPPTPP HLKYLYLVVS DSGISTDYSS GGSQGVHGDS SDGPYSHPYE

491 NSLIPAAEPL PPSYVACS
    ||| |  |||  | |||||
    NSLVPDSEPL HPGYVACS
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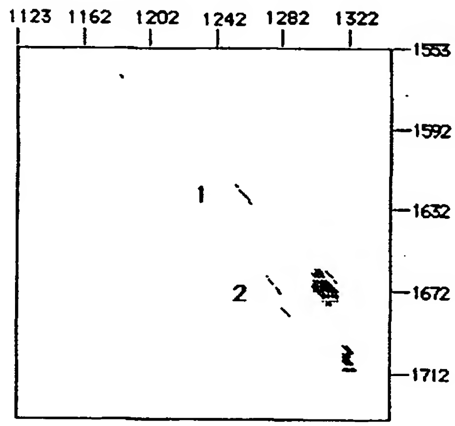
Fig 3. Alignment of human and murine Epo receptor amino acid sequences. The derived polypeptide sequences from the assembled human (HUM) coding sequence and the published murine (MUR) cDNA[4] are shown. The numbers correspond to the human sequence. The one single amino acid gap in alignment (at residue 75) is indicated by a hyphen. The putative transmembrane domain of each is underlined. Vertical lines denote amino acid identity.

was accomplished by nucleotide sequence analysis. Because ER2 does not include the 5′ end of the coding sequence, the remainder has been derived from genomic DNA. Exons were identified by hybridization to 5′ murine Epo receptor sequences. Figure 1 depicts the molecular clones and the nucleotide sequencing stragegy used to analyze ER2 and exons I through III. The assembled coding sequence of the human Epo receptor is shown in Fig 2. The reading frame begins at the underlined ATG. The positions of the 5′ introns are designated by arrowheads. The 22 nucleotides at the 3′ end of exon III are identical to the 5′ end of ER2. The cDNA extends through the translation termination codon (underlined) and the 3′ untranslated region. There are 14 adenine nucleotides at the 3′ end, implying that this is the site of poly(A) addition. The genomic DNA sequence diverges from the cDNA at this point (data not shown), also suggesting that this is the authentic site of polyadenylation. Surprisingly, there is no consensus poly(A) addition signal (AATAAA) upstream of the 3′ end.

Figure 3 displays the alignment of the reported murine receptor[4] with the derived 508 residue human Epo receptor amino acid sequence. The predicted peptide has a calculated molecular weight of 55.24 Kd. There is an 82% amino acid sequence identity between murine and human receptors, with a single amino acid gap in alignment. The putative hydrophobic leader and transmembrane domains of the murine receptor align exactly with that of the human sequence, as determined by hydrophilicity plot (data not shown). The degree of homology is similar throughout the length of the molecule.

As expected, there is a high degree of nucleotide sequence conservation between human and murine Epo receptor coding sequences. Interestingly, there is sequence homology between the 3′ untranslated regions of the murine and human cDNAs. Figure 4A shows a homology matrix plot of the 3′ untranslated sequences. With the parameters used (a search segment of 25 nucleotides, allowing eight mismatches), sequence homology is detected in two discrete segments, designated 1 and 2. Figure 4B shows the sequence alignment of the conserved regions. Segment 1 is 25 bases in length and 76% identical between mice and humans, with no gaps in alignment. A search of nucleotide sequence databanks showed no other genes with close homology to segment 1. Segment 2 is 35 bases long and is 71% identical, with one single nucleotide gap. Segment 2 is just 5′ to the apparent poly(A) tail and includes a stretch of simple (TA) repeats. On

A



B

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1  CTGAGCAGGAAGAGACAGCCTTGCA MURINE
   |||  ||||||    ||| |||||||
   CTGCTCAGGAAACCACAACCTTGCA HUMAN

2  TATCTGTCTGTATATAG-AAATATATATATATATA MURINE
   ||| | | | | | | | |  |||||||||||||
   TATGTAGTTTTTTTTGTATCTATATATATATATA HUMAN
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Fig 4. Homology within the 3′ untranslated regions of human and murine Epo receptor cDNA. (A) Homology matrix analysis of the human and murine cDNA 3′ untranslated sequences. ER2 is on the horizontal axis and murine cDNA is on the vertical axis; the nucleotide numbering is shown. The search segment is 25 nucleotides; the maximum number of mismatches allowed is eight nucleotides. Two short segments of high homology are detected. These are identified as 1 and 2. (B) The homologous segments 1 and 2 are aligned. Vertical lines denote sequence identity. The hyphen shows a single nucleotide gap in alignment.

databank search, many intergenic and intronic regions, as well as viral, mitochrondrial, and protozoan genomes, possess simple (TA) repeats homologous to segment 2, yet very few messenger RNA (mRNA)-encoding regions exhibit such homology. A murine 68-Kd heat shock protein gene (GenBank file MUSHSP68C)[23] and a human homeobox gene (GenBank file HUMHOX329)[24] are the only mammalian genes we could identify with close homology to sequence 2 within transcribed DNA. Interestingly, the sequence is also present in the 3′ untranslated region of these genes.

Human chromosome assignment and sublocalization of the Epo receptor gene has been studied by in situ hybridization, using ER2 as a probe (Fig 5A). Two hundred cells are analyzed in the experiment shown. Forty-two grains (9.7%) localize to 19p, signigicantly higher than the average number of 3.9 per region. This experiment has been performed twice; in the second, 9.5% of grains localize to 19p. In each experiment statistical significance is achieved to $P < .0005$, using Clapham's ratio test for a random distribution.[25]

The chromosome assignment of the human Epo receptor gene has been confirmed by hybridization of labeled ER2 cDNA to a panel of flow-sorted human metaphase chromosomes (Fig 5B). In this experiment, 3×10^4 chromosomes are sorted onto individual spots on a nitrocellulose disc, except chromosomes 9 through 12, which cannot be separated. 1.2×10^5 of the fraction of chromosomes that includes 9 through 12 are on the same spot. Background hybridization is seen over several human chromosomes. This is proportionally higher in the 9 through 12 spot. Nevertheless, the strongest signal is observed over chromosome 19. The irregular spacing of hybridization signals in Fig 5B is due to poor alignment of the filter discs during x-ray exposure.

DISCUSSION

We describe the coding sequence of the gene for the human Epo receptor. As expected, there is a high degree of nucleotide and derived amino acid sequence conservation between the murine and human receptors. If one presumes the murine receptor molecule to be of adult derivation, it is notable that there appears to be no major structural dissimilarity between this molecule and the human fetal liver receptor (encoded by ER2). This would suggest that the relative GM-CSF or IL-3 dependence of Epo action in adult versus fetal RBC precursors[26,27] is determined by other molecules, perhaps interacting with the Epo receptor.

We observe two distinct , short 3′ untranslated region homologies between human and murine Epo receptors (Fig 4). Usually there is no significant sequence conservation between noncoding sequences of mouse and human cDNAs. Several examples exist of such sequence homologies that have important functional implications. Conserved untranslated region sequences are involved in posttranscriptional regulation of gene expression[28-30] and determination of mRNA stability.[31,32] There is no evidence that the conserved 3′ sequence of the Epo receptor is functionally important. The possibility that these sequences may be involved in Epo receptor gene expression remains to be tested experimentally.

In situ hybridization data sublocalize the gene encoding the ER2 cDNA to human chromosome 19p. This assignment is confirmed by the independent technique of hybridization to a panel of sorted human chromosomes. No inherited disorders of human erythropoiesis have been mapped to 19p.[33] Also, this chromosomal region exhibits no extended locus homology with a corresponding mouse chromosome.[34] Interestingly, a human erythroleukemia cell line (TF-1 cells) has several karyotype abnormalities.[35] It has two extra chromosomes 19 that are designated 19p+ (ie, 19p has extra genetic material). TF-1 cells are known to express high levels of Epo receptors.[36] One might speculate that abnormalities in structure or expression in the Epo receptor gene may have a role in the pathogenesis of this erythroleukemia.

The availability of molecular clones for the Epo receptor will allow detailed molecular analysis of normal and pathologic erythropoiesis. Several important questions concerning the receptor itself remain unanswered. First, there are inconsistencies between data obtained from Epo receptor crosslinking experiments[36-43] and the peptide structure derived from cDNA.[4] Second, the determinants of receptor binding affinity are unknown.[3,4,37,41,42,44] Third, the putative

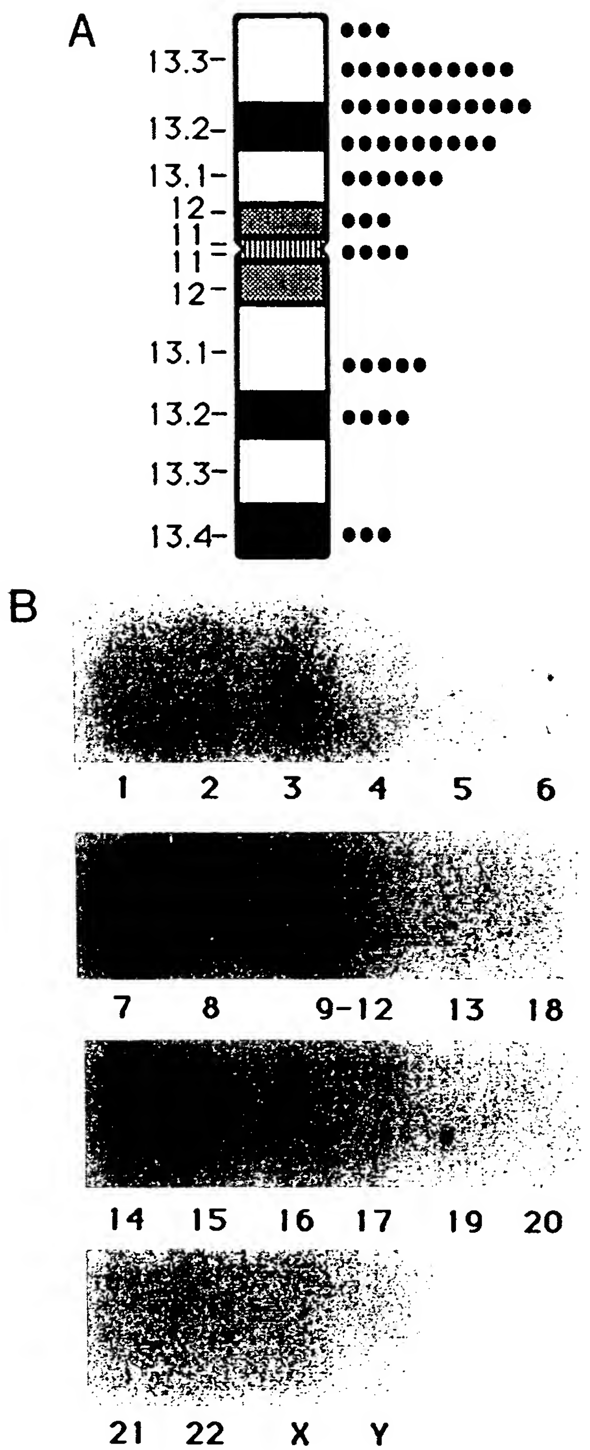

Fig 5. Assignment of the gene for the human Epo receptor to chromosome 19p. (A) In situ hybidization of Epo receptor cDNA to human metaphase chromosomes. A chromosome 19 idiogram is shown that gives the autoradiographic grain distribution in one experiment. Chromosome band positions are designated. In this experiment 200 cells were analyzed. Four hundred thirty-four grains were observed over 110 regions. Forty-two grains were observed over the chromosome 19p-arm ($P < .0005$, Clapham's ratio test for a random distribution[25]). (B) Results of the hybridization of labeled ER2 cDNA to a panel of flow-sorted human metaphase chromosomes. Chromosomes are sorted directly onto nitrocellulose discs and hybridized to labeled ER2 cDNA as described.[20-22] The position of each sorted chromosome is marked on the filter discs. The authenticity of hybridization signals is established by superimposing the autoradiograph on the marked filters. Chromosomes 3×10^4 are placed on each spot, except chromosomes 9 through 12, which cannot be separated. Chromosomes, 1.2×10^5, are placed on the 9 through 12 spot. The strongest signal is easily seen over chromosome 19. The irregular spacing of hybridization signals in (B) is due to poor alignment of the filter discs during x-ray exposure.

interactions of the Epo receptor with other membrane proteins are undefined. The solution of these problems will be an important step in understanding erythropoiesis. In addition, studies to determine the precise regions of the receptor molecule involved in ligand binding and signal transduction are possible. Expression of the Epo receptor gene is likely to be one of the earliest events in hematopoietic commitment to the RBC lineage. Therefore, the regulation of this gene is of keen interest.

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